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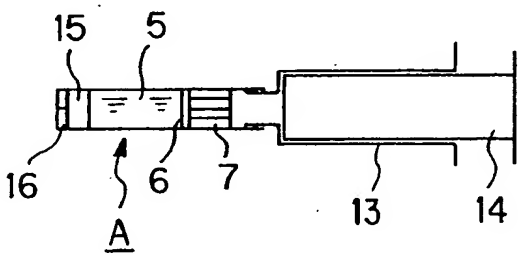
(54) METHOD FOR RAPIDLY DETERMINING NUMBER OF BACTERIA AND EQUIPMENT FOR DETERMINING NUMBER OF BACTERIA

(57) The inventions as set forth in claims 1 to 9 provide methods and equipment for determining the number of bacteria in a sample rapidly and readily in a single filtration operation without necessitating any special facility and special knowledge. The inventions as set forth in claims 10 to 18 provide methods and kits for similarly determining the number of bacteria in a sample rapidly and readily in a single-stage filtration operation using an injection syringe without using any sampling tool such as a dropping pipette. The invention as set forth in claim 1 provides a method for rapidly determining the number of bacteria in a sample characterized by leading a bacterial sample into a tubular filtration vessel which has in its interior a hydrophobic filter for detecting bacteria, a dye solution composition disposed on such a side of the filter as will receive a bacterial sample, and a filter support (and further a piston and an aqueous solution in claim 2) disposed on the opposite side of the filter from the bacterial sample reception side to stain the sample, filtering the stained sample by suction from the support side to simultaneously conduct the trapping of the stained bacteria on the filter and the removal of excess dye, and determining the degree of coloring of the filter. The invention as set forth in claim 10 provides a method for rapidly determining the number of bacteria in a sample characterized by leading a bacterial sample by suction into a filtration vessel having in its interior a hydrophobic filter for detecting bacteria, pushing the sample into a dye solution composition, trapping the stained bacteria on the hydrophobic filter by filtration under suction, and determining the degree of coloring of the filter.

FIG.1



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Description

Technical Field:

This invention as set forth in claims 1 to 9 relates to a method and a device which make it possible to determine the number of bacteria in a sample quickly and easily in a single step of filtering operation without requiring any highly developed skill, or expert knowledge.

This invention as set forth in claims 10 to 18 relates to a method and a kit which make it possible to determine the number of bacteria in a sample quickly and easily in a single step of filtering operation without requiring any highly developed skill, or expert knowledge, since no dropper, or other device is required for taking a sample to be inspected, but a filtering operation under suction with a syringe is sufficient therefore.

This invention as set forth in claims 1 to 18 can be used effectively in a wide variety of fields, such as the field of diagnosis based on bacteria in urine, the field of metal processing fluids, the field of dyes involving a problem of decomposition, the field of food, and the field of environmental problems including that of hot-spring water.

Background Art:

In the field of urine analysis, etc., it has been usual to determine the number of bacteria, etc. in a sample of urine, etc. to discover inflammation and estimate the condition of a disease.

For example, a case involving an increase of bacteria in urine is diagnosed as bacteriuria, and suggests an infection of the urinary tract. Accordingly, the number of bacteria is determined for early treatment and administration of medicine.

In the field of food, etc., the number of bacteria, etc. in food are determined to see if it has not been decomposed, and in the case of fermented milk, lactic-acid beverages, etc., the determination of the number of lactic-acid bacteria in the food is performed to control it.

The determination of the number of bacteria in such cases is mainly performed by a method employing agar plate culture.

This method, however, requires expert skill and an apparatus called an incubator, and calls for 24 to 48 hours to give results.

In the case of urine analysis, for example, it is after several days that a doctor knows the results of an examination by culture of an outpatient's sample of urine which is performed in a bacteriological examination room, or center. A method for the quick determination of bacteria is desired for diagnosis of high accuracy.

Food is examined for Escherichia coli and for general bacteria, and while the results of an examination for Escherichia coli are available from 18 hours of culture, an examination for general bacteria requires 24 or more hours of culture, and the results thereof are usually available for many foods only after their shipment. An improvement has been desired for bacterial control in the determination of, among others, the number of lactic-acid bacteria in fermented milk, etc., since it usually requires about 72 hours of culture, and the number of lactic-acid bacteria is known only after the shipment of commercial products, and in many cases after consumers have drunk or eaten the products.

In the field of metal processing fluids, such as water-soluble cutting fluids, the growth of bacteria is likely to cause the decomposition of fluids. An easy culture kit (such as one sold under the tradename, Easicult) is presently available for checking the presence of bacteria, but takes 48 hours to give results. Therefore, it is likely that the measures taken for preventing decomposition may be too late to prevent any objectionable odor from being produced by decomposition.

Moreover, there have been proposed a method of determining the enzyme activity of bacteria (Japanese Patent Application Laid-Open No. Sho 57-74095), a method using a fluorescent dye (Japanese Patent Application Laid-Open No. Sho 62-138185), a filter dyeing method (Japanese Patent Application Laid-Open No. Hei 1-124767), etc., though they are not presently employed in practice, but they have revealed drawbacks, such as the instability of a reagent used for determining enzyme activity, the necessity for a special apparatus (fluorophotometer), and the complicated operation involved in the dyeing process.

The applicant of this application has already proposed a method for determining the number of microorganisms in a sample by either dyeing the microorganisms and collecting them in a hydrophobic filter, or collecting them in a hydrophobic filter and dyeing them, washing away the excess of coloring matter and determining the number of the microorganisms in the sample from the degree of their staining, as a method overcoming the drawbacks of the prior art as stated above (Japanese Patent Application Laid-Open No. Hei 4-218392).

This method has made it possible to determine the number of microorganisms in a sample quickly and easily without requiring any highly developed skill, expert knowledge, or special equipment.

The actual use of the method has, however, revealed that it is difficult and complicated, since it requires two steps of filtering operation.

The necessity for the use of a dropper, or like device for taking a sample has also complicated the method.

It is an object of this invention as set forth in claims 1 to 9 to overcome the drawbacks of the prior art as stated above, and provide a method and a device which make it possible to determine the number of bacteria in a sample quickly (within one minute) and easily in a single step of filtering operation without requiring any special equipment, or expert knowledge.

It is an object of this invention as set forth in claims 10 to 18 to provide a method and a kit which make it possible to determine the number of bacteria in a sample quickly (within one minute) and easily in a single step of filtering operation without requiring any special equipment, or expert knowledge, since no dropper, or like device is used for taking a sample, but a filtering operation under suction with a syringe is sufficient therefore.

Disclosure of the Invention:

This invention as set forth in claim 1 provides a method of determining the number of bacteria in a sample quickly which comprises introducing a sample containing bacteria into a tubular filtering vessel holding therein a hydrophobic filter for bacterial detection, a coloring composition on that side of the filter where the sample is introduced into the vessel, and a support for the filter on the opposite side of the filter from the coloring composition, dyeing the bacteria, filtering the sample by suction from the support to collect the dyed bacteria on the filter and remove the excess of coloring matter, and determining the number of the bacteria in the sample from the degree of staining of the filter.

This invention as set forth in claim 2 provides a method of determining the number of bacteria in a sample quickly which comprises introducing a sample containing bacteria into a tubular filtering vessel holding therein a hydrophobic filter for bacterial detection, a coloring composition on that side of the filter where the sample is introduced into the vessel, and a support for the filter, a piston and an aqueous solution on the opposite side of the filter from the coloring composition, dyeing the bacteria, filtering the sample by moving the piston in the aqueous solution in the vessel to collect the dyed bacteria on the filter and remove the excess of coloring matter, and determining the number of the bacteria in the sample from the degree of staining of the filter.

The invention as set forth in claim 2 is characterized by including the piston and the aqueous solution in the filtering vessel according to the invention as set forth in claim 1.

This invention as set forth in claim 8 provides a device for determining the number of bacteria which comprises a tubular filtering vessel having a sample inlet at one end, while the other end thereof enables suction with a syringe, the vessel holding a coloring composition, a hydrophobic filter for bacterial detection and a filter support therein in their order as viewed from the sample inlet.

This invention as set forth in claim 9 provides a device for determining the number of bacteria which comprises a tubular filtering vessel having a slender sample introducing tube connected at one end, while the other end thereof enables suction with a syringe, the vessel holding a prefilter, a coloring composition, a hydrophobic filter for bacterial detection, a filter support, a piston movable in the vessel, an aqueous solution and a plug preventing the leakage of the aqueous solution therein in their order as viewed from the slender tube.

The invention as set forth in claim 9 is characterized by including the prefilter, piston movable in the vessel, aqueous solution, and plug preventing the leakage of the aqueous solution in the filtering vessel according to the invention as set forth in claim 8.

This invention as set forth in claim 10 provides a method of determining the number of bacteria in a sample quickly which comprises introducing a sample containing bacteria by suction into a filtering vessel holding a hydrophobic filter for bacterial detection therein, extruding it into a coloring composition, filtering it by suction to collect the dyed bacteria on the filter and determining the number of the bacteria in the sample from the degree of staining of the filter.

This invention as set forth in claim 17 provides a kit for determining the number of bacteria which comprises a filtering vessel holding a hydrophobic filter for bacterial detection and a filter support therein, a sampling member containing a prefilter and adapted for connection to one end of the vessel, a coloring composition, a vessel for the coloring composition and a color reference chart.

This invention as set forth in claim 18 provides a kit for determining the number of bacteria which comprises a filtering vessel holding a hydrophobic filter for bacterial detection and a filter support therein, a sampling member adapted for connection to one end of the vessel, a coloring composition, a vessel for the coloring composition and a color reference chart.

The invention as set forth in claim 18 differs from the invention as set forth in claim 17 in that the sampling member does not contain any prefilter.

Brief Description of the Drawings:

Figure 1 is an explanatory view showing one form of a device of this invention for determining the number of bacteria as set forth in claim 8.

Figure 2 is an explanatory view showing one form of a device of this invention for determining the number of bac-

teria as set forth in claim 9.

Figure 3 is an explanatory view showing the mode in which a sample is introduced into the device of this invention for determining the number of bacteria shown in Figure 2 by a syringe attached thereto.

Figure 4 ((a) to (f)) shows the manner in which the device of this invention for determining the number of bacteria is used.

In Figures 1 to 4, symbol A denotes a filtering vessel, symbol 1 a slender sampling tube, symbol 2 a sampling scale, symbol 3 a severing mark, symbol 4 a prefilter, symbol 5 a coloring composition, symbol 6 a hydrophobic filter for bacterial detection, symbol 7 a filter support, symbol 8 a piston, symbol 9 an aqueous solution, symbol 10 a tubular insert, symbol 11 a tubular insert, symbol 12 a plug, symbol 13 a syringe, symbol 14 a plunger, symbol 15 a sample inlet, and symbol 16 a plug.

Figure 5 is an explanatory view showing one form of a kit of this invention for determining the number of bacteria as set forth in claim 17.

Figure 6 is an explanatory view showing a combination of a kit of this invention for determining the number of bacteria as set forth in claim 17 and a commercially available syringe which is ready for determining the number of bacteria.

In Figures 5 and 6, symbol A denotes a filtering vessel, symbol B a syringe, symbol C a sampling member, symbol D a coloring composition, symbol E a vessel for the coloring composition, symbol F a color reference chart, symbol 21 a hydrophobic filter for bacterial detection, symbol 22 a filter support, symbol 23 a prefilter, symbol 24 a sample inlet, symbol 25 a dropping bottle, and symbol 26 a piston.

Best Mode of Carrying Out the Invention:

The invention as set forth in claim 1 and the invention as set forth in claim 10 are basically different from each other in whether the coloring composition is held in the filtering vessel, or not.

The method of this invention as set forth in claim 1 is preferably carried out by employing the device of this invention as set forth in claim 8. The method of this invention as set forth in claim 2 is preferably carried out by employing the device of this invention as set forth in claim 9.

Therefore, description will now be made of the method of this invention as set forth in claim 1 with reference to the device of this invention as set forth in claim 8, and then of the method of this invention as set forth in claim 2 with reference to the device of this invention as set forth in claim 9.

Referring first to the method of this invention as set forth in claim 1, a sample containing bacteria (for example, fermented milk, or a lactic-acid beverage) is introduced into a tubular filtering vessel holding therein a hydrophobic filter for bacterial detection, a coloring composition on that side of the filter where the sample is introduced (on the left-hand side of the filter in Figure 1 as will be described) and a support for the filter on the opposite side of the filter from the coloring composition (on the right-hand side of the filter in Figure 1 as will be described), and is dyed before the number of bacteria in the sample is determined.

The filtering vessel is tubular, or may be cylindrical or tubular, and may be of either plastics, such as vinyl chloride, or glass. Its capacity depends on the amount of the coloring composition, etc. as employed.

The filtering vessel holds therein a hydrophobic filter, a coloring composition on that side of the filter where the sample is introduced, and a support for the filter on the opposite side of the filter from the coloring composition. The filtering vessel is preferably as set forth in claim 8.

Referring now to the method of this invention as set forth in claim 2, a sample containing bacteria is introduced into a tubular filtering vessel holding therein a hydrophobic filter for bacterial detection, a coloring composition on that side of the filter where the sample is introduced (on the left-hand side of the filter in Figure 2 as will be described) and a support for the filter, a piston and an aqueous solution on the opposite side of the filter from the coloring composition (on the right-hand side of the filter in Figure 2 as will be described), and is dyed before the number of bacteria in the sample is determined.

The filtering vessel according to the invention as set forth in claim 2 is tubular, or may be cylindrical or tubular, and may be of either plastics, such as vinyl chloride, or glass, as is the case with the invention as set forth in claim 1. Its capacity depends on the amount of the coloring composition, etc. as employed. The filtering vessel holds therein a hydrophobic filter for bacterial detection, a coloring composition on that side of the filter where the sample is introduced, and a support for the filter, a piston and an aqueous solution on the opposite side of the filter from the coloring composition. The filtering vessel is preferably as set forth in claim 9.

The invention will now be described with reference to the drawings. Figure 1 is an explanatory view showing one form of a device of this invention for determining the number of bacteria as set forth in claim 8. In the drawing, symbol A denotes a filtering vessel.

The filtering vessel A in the device for determining the number of bacteria as set forth in claim 8 is a tubular one having a sample inlet 15 at one end, while the other end thereof enables suction with a syringe 13, and the filtering vessel A holds a coloring composition 5, a hydrophobic filter 6 for bacterial detection and a filter support 7 therein in their

order as viewed from the sample inlet 15. The syringe 13 can be included in the device of this invention as set forth in claim 8, if required.

The sample inlet 15 at one end of the filtering vessel A has an end which may be left open, or may be provided with an appropriate plug 16 for preventing the leakage of the coloring composition 5. The plug 16 may be replaced by a tubular insert, or the like.

The other end of the filtering vessel A enables suction with the syringe 13 which can be connected thereto at one end. The syringe 13 itself can, however, be included in the device of this invention, if required, as stated above.

The coloring composition 5 held in the filtering vessel A is a composition which can be used for both dyeing and washing, and more specifically, it comprises coloring matter which can be used for dyeing bacteria, and water, or a buffer solution.

Examples of the coloring matter which can be used for dyeing bacteria are Safranine, Fuchsine, Methylene Blue, Methyl Green, Crystal Violet, Gentiana Violet and Victoria Blue B, and Safranine, Fuchsine, Methylene Blue or Methyl Green is preferred from the standpoint of easy judgment.

The buffer solution which can be used has a pH of 4 to 9 and preferably has a pH of 5 to 7 to ensure the stability of the coloring matter. A preservative, such as ethanol, a surface active agent, etc. can be added, if required.

The concentration of the coloring matter is usually from 0.00001 to 0.00045% (w/v) and preferably from 0.00015 to 0.0004% (w/v) when it is Fuchsine. If its concentration is less than 0.00001%, it is insufficient for any satisfactory coloring, and if its concentration exceeds 0.00045%, it is difficult to remove any excess of the coloring matter.

If it is necessary to add a preservative, such as ethanol, it can be added in the amount of 0.1 to 15% (w/v), and if a surface active agent is added, it can be added in the proportion of from 0.001 to 1.0% (w/v), and preferably from 0.01 to 0.6% (w/v).

The adequate amount of the coloring composition to be used per sample is two to five times the amount of the sample. The amount of each sample is usually 50 to 100 μl. Therefore, any sample in the amount of 100 μl requires 200 to 500 μl of coloring composition. No amount exceeding five times that of the sample is, however, required.

It is preferable for preventing any error to use the same amount of the coloring composition when preparing a color reference chart or a working curve as when applying it to any sample containing an unknown number of bacteria.

The filtering vessel A holds the hydrophobic filter 6 for bacterial detection after the coloring composition 5 as viewed from the sample inlet 15. The method of this invention employs a hydrophobic filter 6 as a filter for bacterial detection. The hydrophobic filter is suitable as a filter for collecting bacteria, since it is of low or no polarity, and it is easy to remove any excess of coloring matter therefrom. A polar filter is undesirable, as it is not easy to remove any excess of coloring matter therefrom.

The hydrophobic filter 6 is of, for example, any of nylons, fluororesins such as polytetrafluoroethylene, polyolefins such as polypropylene, polycarbonates, and glass. A hydrophobic filter made of polytetrafluoroethylene or polypropylene is, among others, preferred, as it is easy to remove any excess of coloring matter.

A filter of a hydrophilic material, such as nitrocellulose, can be used as a hydrophobic filter if its surface is made hydrophobic by surface treatment. Its surface treatment is, for example, its coating with any of the materials including nylons, fluororesins and polyolefins as mentioned above.

The hydrophobic filter 6 for bacterial detection has a pore diameter of, say, 0.2 to 3.0 μm which is usually adopted for a filter for collecting bacteria.

The hydrophobic filter 6 has an area of filtration by suction (a coloring zone) which is not particularly limited in size if it is easily visible. The coloring zone is not particularly limited in shape, but in order to be easily visible, it is preferably circular with a diameter of, say, 1 to 3 mm. There is no particular limitation in the size of the hydrophobic filter 6 as a whole, either, though it is at least equal to the size of the coloring zone.

The hydrophobic filter 6 may have a color so selected as to facilitate judgment by taking into consideration the coloring composition to be used. A white hydrophobic filter is preferred to facilitate judgment on the degree of coloring. A transparent or semi-transparent hydrophobic filter can also be employed, and can be placed on white paper to facilitate judgment.

The filtering vessel A holds the filter support 7 after the coloring composition 5 and the hydrophobic filter 6 for bacterial detection as viewed from the sample inlet 15. The filter support 7 may be of any material and shape if it can support the hydrophobic filter 6. It is usually formed from a silicone resin, or the like.

The filtering vessel A in the device for determining the number of bacteria as set forth in claim 8 is a tubular one having the sample inlet 15 at one end, while the other end thereof enables suction with the syringe 13, as stated above, and the filtering vessel A holds the coloring composition 5, the hydrophobic filter 6 for bacterial detection and the filter support 7 therein in their order as viewed from the sample inlet 15.

The device of this invention for determining the number of bacteria as set forth in claim 8 is of the construction as described above.

According to the method as set forth in claim 1, an appropriate sample containing bacteria, preferably such as fermented milk, or a lactic-acid beverage, is introduced into the filtering vessel A, and dyed.

There is no particular limitation to the sample to which this invention is applicable, but it is applicable to a wide variety of samples including not only urine, but also water-soluble metal processing fluids such as cutting, rolling and heat-treatment fluids, liquid foods such as liquid seasonings (soy, sauce, etc.), liquid foods and beverages (fermented milk, lactic-acid beverages, etc.) and alcoholic drinks (wine, sake, etc.), samples left after washing powdery, or solid foods (vegetables, fish, meat, etc.), water-soluble paints, and water, such as river, pond or hot-spring water, water in a water tank, or waste water from houses. The sample can be appropriately diluted as required, or can be crushed and diluted if it is of, for example, a solid food.

The method as set forth in claim 1 is intended for determining quickly the total number of bacteria existing in any such sample, for example, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa.

The inventions as set forth in claims 1 and 8 are particularly useful for determining the number of lactic-acid bacteria in fermented milk and lactic-acid beverages quickly.

Description will now be made of the process in which a sample containing bacteria as mentioned above is placed in the filtering vessel A, and dyed in accordance with the invention as set forth in claim 1.

The syringe 13 is attached to the opposite end of the filtering vessel A from its sample inlet 15 (though this step is unnecessary if the syringe is already set in position), and a sample containing bacteria is introduced through the sample inlet 15, and dyed.

More specifically, the sample is dropped into the coloring composition 5 through the sample inlet 15 by means of, for example, a dropper, and is thoroughly mixed with the coloring composition 5, and thereby dyed. If the plug 16 exists adjacent to the sample inlet 15, the plug 16 is removed before the sample is dropped into the coloring composition 5 by means of a dropper, or the like. If an inserted tube exists instead of the plug 16 adjacent to the sample inlet, a dropper can advantageously be passed through the center of the tube to drop the sample into the coloring composition 5.

Then, the plunger 14 of the syringe 13 is pulled to cause filtration by suction (by drawing air through the filter support 7), whereby the collection of the dyed bacteria on the hydrophobic filter 6 and the removal of any excess of coloring matter are simultaneously carried out. The suction caused by pulling the plunger 14 of the syringe 13 enables a solution containing the sample as dyed (a mixture of the sample and the coloring composition 5) to be collected on the hydrophobic filter 6 simultaneously with the removal of the excess of coloring matter.

As it enables the collection of the dyed bacteria on the hydrophobic filter and the removal of the excess of coloring matter to be carried out simultaneously, the method of this invention as set forth in claim 1 is a simple process.

According to the method of this invention as set forth in claim 1, the collection of the dyed bacteria on the hydrophobic filter and the removal of the excess of coloring matter are carried out simultaneously, as described, for determining the number of the bacteria in the sample from the degree of staining on the filter.

The number of bacteria in the sample is determined from the degree of staining of the bacteria in the sample from which the excess of coloring matter has been removed, as stated above.

For determining the number of bacteria, (1) the simplest way is visual comparison with a color reference chart, but (2) it is also possible to employ colorimetric analysis by measuring optical density. Visual judgment can be made by examining the degree of staining not only on the front side of the hydrophobic filter, but also on its rear side. For this purpose, it is possible to prepare for each side a color reference chart and a working curve showing absorbance and the number of bacteria.

The visual determination of the number of bacteria as stated at (1) above can more specifically be carried out by comparing the degree of staining of bacteria on the hydrophobic filter, or the intensity of their color with a color reference chart prepared by employing samples containing known numbers of bacteria.

The color reference chart can be prepared by taking color photographs of a hydrophobic filter dyed and washed by employing samples containing know numbers of bacteria in accordance with the method as set forth in claim 1, or by coloring filter paper, or the like to the same degree with any such dyed filter, or by printing colors of the same degree on paper.

The following is a description of a color reference chart which is prepared for determining the number of bacteria in urine. Although even the urine of a healthy person contains bacteria at a low concentration [at most about 10,000 per ml (milliliter)], the presence of 100,000 or more per ml is suspected as a case of bacteriuria, and the presence of 1,000,000 or more per ml is definitely concluded as a case of a bacterial infection (bacteriuria). It is, therefore, useful to prepare a color reference chart which enables determination in the vicinity of these values. It is usually sufficient to prepare a standard color reference chart for five levels, i.e., 1,000 per ml, 10,000 per ml, 100,000 per ml, 1,000,000 per ml and 10,000,000 per ml, though this is not intended to be limitative.

The colorimetric analysis based on optical density (O.D.) as stated at (2) above may be carried out by dissolving in an organic solvent the coloring matter which has stained the bacteria on the hydrophobic filter, determining the degree of staining of the resulting solution from its absorbance and comparing it with a working curve prepared beforehand and showing optical density (O.D.) and the number of bacteria. The wavelength employed for measuring absorbance depends on the coloring matter used. Any of alcohols can be used as the organic solvent, but ethanol is, among others, preferred.

A working curve can be prepared as will now be stated by way of example. The degrees of staining of diluted samples having different concentrations are determined by using a microplate reader as optical density (O.D.) at 492 nm if Fuchsine is used as coloring matter, while the numbers of bacteria in the solutions of the same samples as the diluted ones are obtained by counting the number of colonies grown by agar plate culture, and the results of both are combined to form a working curve showing the number of bacteria and optical density.

The invention as set forth in claim 2 will now be described based on the drawings and with reference to the invention as set forth in claim 9.

Figure 2 is an explanatory view showing one form of device of this invention for determining the number of bacteria as set forth in claim 9. Figure 3 is an explanatory view showing the mode in which a sample is introduced into the device shown in Figure 2 by a syringe attached thereto. Figure 4 (Figure 4 (a) to (f)) shows the manner in which the device is used. In the drawings, symbol A denotes a filtering vessel.

The filtering vessel A in the device for determining the number of bacteria as set forth in claim 9 is a tubular one having a slender sampling tube 1 attached to one end thereof, while the other end thereof enables suction with a syringe 13, and the filtering vessel A holds a prefilter 4, a coloring composition 5, a hydrophobic filter 6 for bacterial detection, a filter support 7, a piston 8 movable in the filtering vessel, an aqueous solution 9 and a plug 12 for preventing the leakage of the aqueous solution therein in their order as viewed from the slender tube 1. The syringe 13 can be included in the device of this invention, if required.

The slender sampling tube 1 attached to one end of the filtering vessel A is formed from a flexible material, for example, a polyethylene, or like resin, and usually has a sampling scale 2 marked in its mid-portion for showing a standard amount of a sample. The scale may cover a range of 50 to 100 μl which is the amount of a sample which is usually employed. The slender sampling tube 1 has a distal end which is usually closed to avoid contamination, though it may alternatively be left open, and which is preferably provided with a severing mark 3, as shown.

The other end of the filtering vessel A enables suction with the syringe 13 which can be connected thereto at one end. The syringe 13 itself can, however, be included in the device of this invention as set forth in claim 9, if required, as stated above.

The filtering vessel A first holds the prefilter 4 as viewed from the slender tube 1. The prefilter 4 is used for removing animal cells, and if a sample contains animal cells larger than bacteria, for example, leukocytes, it does not pass any such cells, but collects them, while passing bacteria. The prefilter 4 may have a pore diameter of, say, 6 to 8 microns. It is possible to use as the prefilter a polypropylene filter (having a pore diameter of, say, 7 microns), a urine sampling filter (made of vinyl chloride) for a medicine for a diagnosis for pregnancy, such as "Gonavislide and New Gonavislide" (products of Mochida Pharmaceutical Co., Ltd.) which will appear in the description of examples, a felt, or nonwoven fabric used in a commercially available oily-ink pen, or the like.

The filtering vessel A holds the coloring composition 5 after the prefilter 4 as viewed from the slender tube 1. A tubular insert 10 is usually disposed between the prefilter 4 and the coloring composition 5 for avoiding their contact. The tubular insert 10 is formed from a synthetic resin, such as a silicone resin, and is provided for preventing any contact between the prefilter 4 and the coloring composition 5 prior to use, though it allows a sample containing bacteria to be drawn into the coloring composition 5 through the prefilter 4 upon suction with the syringe.

The filtering vessel A holds the hydrophobic filter 6 for bacterial detection after the prefilter 4 and the coloring composition 5 as viewed from the slender tube 1. The hydrophobic filter 6 duplicates that which has been described in the description of the inventions as set forth in claims 1 and 8.

The filtering vessel A holds the filter support 7, piston 8 movable in the filtering vessel, aqueous solution 9, and plug 12 for preventing the leakage of the aqueous solution in their order after the prefilter 4, coloring composition 5, and hydrophobic filter 6 as viewed from the slender tube 1.

The filter support 7 may be of any material and shape if it can support the hydrophobic filter 6. It is usually formed from a silicone resin, or the like.

The piston 8 movable in the filtering vessel has an outside diameter slightly smaller than the inside diameter of the filtering vessel A, and is slidable in the filtering vessel A as a result of suction, or pressure application with the syringe 13. Although the material of the piston 8 is not particularly limited, a polyacrylamide gel, or konjak (devil's-tongue) is, for example, preferred because of its light weight and low cost.

The aqueous solution 9, as well as the piston 8, isolates the syringe 13 to prevent any sample from entering the syringe 13 and protect the syringe 13 from any bacterial contamination. Accordingly, it is usually possible to use, for example, water, or an aqueous solution containing alcohol. The amount of the aqueous solution is usually, say, 500 to 1000 μl, though it depends on the shape and size of the filtering vessel, the amount of the dyeing solution, etc.

The plug 12 for preventing the leakage of the aqueous solution 9 is held at that end of the filtering vessel A to which the syringe 13 can be attached, and the plug 12 is removed when the syringe 13 is attached, as shown in Figure 3. The plug 12 may be of any material and shape if it functions as a plug for preventing the leakage of the aqueous solution 9.

A tubular insert 11 is usually held inwardly of the plug 12 to ensure the effective avoidance of any leakage of the aqueous solution 9. The tubular insert 11 is formed from a synthetic resin, such as a silicone resin, like the tubular insert

10 as described above, and effectively prevents any leakage of the aqueous solution 9 when the plug 12 has been removed. The filtering vessel A in the device for determining the number of bacteria as set forth in claim 9 is of the construction as described above.

According to the method as set forth in claim 2, a sample containing bacteria is introduced into the filtering vessel A, and dyed.

The invention as set forth in claim 2 is applicable to any samples not particularly limited, but including those stated in the description of the invention as set forth in claim 1. The method as set forth in claim 1 and the device as set forth in claim 8 are simpler, and more suitable for determining the number of lactic-acid bacteria in fermented milk.

Description will now be made with reference to Figure 4 of the process in which a sample containing bacteria as mentioned above is placed in the filtering vessel A, and dyed.

The syringe 13 is attached to the opposite end of the filtering vessel A from the sampling tube 1 (though this step is unnecessary if the syringe is already set in position), and after the sampling tube 1 has had its distal end cut off at the severing mark 3, the filtering vessel A is lowered from its horizontal position at its end connected to the syringe 13 to take a sample, as shown in Figure 4(a).

After the sampling tube 1 has been lifted from the sample solution, the plunger 14 of the syringe 13 is pulled to draw the sample into the filtering vessel A, or more specifically, into the area above the coloring composition 5 (or the area close to the sampling tube 1), as shown in Figure 4(b).

Then, the plunger 14 of the syringe 13 is pushed (to apply pressure) to push the coloring composition 5 back to its original position, as shown in Figure 4(c). On this occasion, the air drawn in with the sample is forced out.

Then, the vessel is raised from its horizontal position at its end connected to the syringe 13 to move bubbles, as shown in Figure 4(d), and thereafter lowered from its horizontal position to move the bubbles to their original position, as shown in Figure 4(e). This movement of bubbles causes the sample and the coloring composition 5 to mix, whereby the sample is dyed. This step is usually taken at least once.

After the sample has been dyed as described, the piston 8 is moved in the aqueous solution 9 in the filtering vessel A for filtration, whereby the collection of the dyed bacteria on the hydrophobic filter 6 and the removal of any excess of coloring matter are carried out simultaneously.

The method of this invention as set forth in claims 1 to 7 enables a simple operation, since it is possible to carry out the collection of the dyed bacteria on the hydrophobic filter and the removal of any excess of coloring matter simultaneously.

The step of filtration by the movement of the piston 8 in the aqueous solution 9 in the filtering vessel A is as shown in Figure 4(f), and if the plunger 14 of the syringe 13 is pulled from the position shown in Figure 4(e), the piston 8 moves in the aqueous solution 9 in the filtering vessel A, whereby a solution containing the dyed sample (a mixture of the sample solution and the coloring composition 5) is collected on the hydrophobic filter 6, while any excess of coloring matter is simultaneously removed.

After the collection of the dyed bacteria on the hydrophobic filter and the removal of any excess of coloring matter has been carried out simultaneously as described, the number of bacteria in the sample is determined from the degree of staining of the filter, as is the case with the invention as set forth in claim 1.

Description will now be made of the invention as set forth in claims 10 to 18. The method of this invention as set forth in claim 10 is preferably carried out by employing a kit of this invention for determining the number of bacteria as set forth in claim 17 or 18. Accordingly, the method of this invention as set forth in claim 10 will be described with reference to the kit of this invention as set forth in claim 17 or 18.

When the method of this invention as set forth in claim 10 is used for determining the number of bacteria in a sample, a sample containing bacteria (for example, fermented milk, or a fermented milk beverage) is drawn into a filtering vessel holding a hydrophobic filter for bacterial detection therein.

The filtering vessel duplicates that which has been described in connection with the invention as set forth in claim 1, etc. More specifically, the filtering vessel is tubular, or may be cylindrical or tubular, and may be of either plastics, such as vinyl chloride, or glass. Its capacity depends on the amount of the coloring composition, etc. as employed. The filtering vessel holds therein a hydrophobic filter for bacterial detection, and a support for the filter. The filtering vessel is preferably as set forth in claim 8, 9 or 17.

The invention as set forth in claims 10 to 18 will now be described with reference to the drawings. Figure 5 is an explanatory view showing one form of a kit of this invention for determining the number of bacteria as set forth in claim 17. Figure 6 is an explanatory view of a combination of the kit of this invention as set forth in claim 17 and a commercially available syringe which is ready for determining the number of bacteria. In the drawings, symbol A denotes a filtering vessel.

The filtering vessel A in the kit as set forth in claim 17 holds a hydrophobic filter 21 for bacterial detection and a filter support 22 therein. The filtering vessel A is tubular, or may be cylindrical or tubular, and holds the hydrophobic filter 21 at one end thereof. The filtering vessel A may be of either plastics, such as vinyl chloride, or glass, but is preferably of plastics owing to its ease of handling.

The method of this invention as set forth in claim 10 employs a hydrophobic filter 21 as a filter for bacterial detection. The hydrophobic filter 21 may be equal to the hydrophobic filter 6 as described in connection with the invention as set forth in claim 1 (reference is made to the foregoing description for details on the hydrophobic filter, such as its material, pore diameter, and area of filtration under suction). The hydrophobic filter 21 is preferably made of polytetrafluoroethylene, or polypropylene, since it is easy to remove any excess of coloring material, as is the case with the invention as set forth in claim 1.

The filter support 22 may be of any material and shape if it can support the hydrophobic filter 21. It is usually formed from a silicone resin, or the like.

The other end of the filtering vessel A enables suction with a syringe B which can be attached to it at one end. The syringe B itself can be included in the kit of this invention as set forth in claim 17, if required.

A sampling member C holding a prefilter 23 therein is detachably combined with the filtering vessel A at its end (at which it holds the hydrophobic filter 21), as shown in Figure 5. The filtering vessel A and the sampling member C are usually so constructed that the filtering vessel A may fit at one end in the sampling member C and have its hydrophobic filter 21 contact the prefilter 23 in the sampling member C, as shown in Figures 5 and 6.

The sampling member C is tubular, or may be cylindrical or tubular, and has a tapered end provided with a sample inlet 24 through which it can take a sample. The sampling member C preferably has measuring graduations. Figure 5 shows the sampling member C as having three graduations. The graduations may cover a range of 50 to 100 μl which is the amount of a sample which is usually employed.

The prefilter 23 in the sampling member C may be equal to the prefilter 4 as described in connection with the invention as set forth in claim 1. The prefilter 23 is used for removing animal cells, and if a sample containing bacteria contains also animal cells larger than bacteria, such as leukocytes, it does not pass any such animal cells, but collects them, while passing bacteria. The prefilter 23 can, therefore, be omitted, as is obvious from claim 18, if the sample is of, for example, fermented milk, or a fermented milk beverage, and is free from any such animal cells.

The prefilter 23 may have a pore diameter of, say, 6 to 60 μm. It is possible to use as the prefilter a polypropylene filter (having a pore diameter of, say, 7 μm), a urine sampling filter (made of vinyl chloride) for a medicine for a diagnosis for pregnancy, such as " Gonavislide and New Gonavislide" (products of Mochida Pharmaceutical Co., Ltd.) which will appear in the description of examples, a felt, or nonwoven fabric used in a commercially available oily-ink pen, or the like.

When the invention as set forth in claim 10 is used for determining the number of bacteria in a sample, the sample (for example, fermented milk, or a fermented milk beverage) is first drawn by suction into the filtering vessel A holding the hydrophobic filter 21 for bacterial detection therein, and this step of suction will now be described.

The sampling member C holding the prefilter 23 therein is attached to (or fitted on) the end of the filtering vessel A (at which it holds the hydrophobic filter 21). They are so put together that the prefilter 23 may contact the hydrophobic filter 21. It is, however, alternatively possible to employ a sampling member C having no prefilter 23 as set forth in claim 18.

Then, the syringe B is attached to the other end of the filtering vessel A (the opposite end thereof from the hydrophobic filter 21) to get ready for suction. These steps can, however, be reversed in order.

Figure 6 shows the assembly which is ready for the step of suction. An appropriate amount of a sample containing bacteria is drawn by suction with the syringe B into the sampling member C through the sample inlet 24 at its distal end. The measuring graduations on the sampling member C provide a standard for the amount of the sample.

The samples to which the invention as set forth in claim 10 is applicable include those listed above in the description of the invention as set forth in claim 1. More specifically, it is applicable to a wide variety of samples including not only urine, but also water-soluble metal processing fluids such as cutting, rolling and heat-treatment fluids, liquid foods such as liquid seasonings (soy, sauce, etc.), liquid foods and beverages (fermented milk, lactic-acid beverages, etc.) and alcoholic drinks (wine, sake, etc.), samples left after washing powdery, or solid foods (vegetables, fish, meat, etc.), water-soluble paints, and water, such as river, pond or hot-spring water, water in a water tank, or waste water from houses.

The method as set forth in claim 10 is intended for determining quickly the total number of bacteria existing in any such sample, for example, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa.

The kit of this invention as set forth in claims 18 is particularly useful for determining the number of bacteria in fermented milk and fermented-milk beverages quickly.

The sample which has been drawn in by suction with the syringe B as described above is extruded into a coloring composition D. The coloring composition D is held in an appropriate container (for example, a dropping bottle 25) forming a part of the kit, as shown in Figure 5, and the kit further includes another appropriate container E capable of holding that amount of the coloring composition D which is necessary for about a single step of filtration by suction, for example, a microtube having a capacity of, say, 0.5 to 1.5 ml, so that it may hold the coloring composition D for actual use in determining the number of bacteria.

A small amount of coloring composition D is fed from the dropping bottle 25, or the like into the container E (such

as a microtube), and the sample drawn into the syringe B is extruded for injection into the coloring composition, and is thoroughly mixed with it. As a result, the sample is dyed with the coloring composition D.

The coloring composition D is a composition which can be used for both dyeing and washing, and more specifically, it comprises coloring matter which can be used for dyeing bacteria, and water, or a buffer solution, and a surface active agent, if required.

Examples of the coloring matter which can be used for dyeing bacteria in accordance with the invention as set forth in claim 10 include those listed above in the description of the invention as set forth in claim 1. Safranine, Fuchsine, Methylene Blue or Methyl Green is preferred from the standpoint of easy judgment, as is the case with the invention as set forth in claim 1.

The buffer solution which can be used has a pH of 4 to 9 and preferably has a pH of 5 to 7 to ensure the stability of the coloring matter. A preservative, such as ethanol, a surface active agent, etc. can be added, if required.

The concentration of the coloring matter is usually from 0.00001 to 0.00045% (w/v) and preferably from 0.00015 to 0.0004% (w/v) when it is Fuchsine. If its concentration is less than 0.00001%, it is insufficient for any satisfactory coloring, and if its concentration exceeds 0.00045%, it is difficult to remove any excess of the coloring matter.

If it is necessary to add a preservative, such as ethanol, it can be added in the amount of 0.1 to 15% (v/v). If a surface active agent is added, it can be added in the proportion of from 0.001 to 1.0% (w/v), and preferably from 0.01 to 0.6% (w/v).

The adequate amount of the coloring composition to be used per sample is two to five times the amount of the sample. The amount of each sample is usually 50 to 100 μl. Therefore, any sample in the amount of 100 μl requires 200 to 500 μl of coloring composition. No amount exceeding five times that of the sample is, however, required.

It is preferable for preventing any error to use the same amount of the coloring composition when preparing a color reference chart or a working curve as when applying it to any sample containing an unknown number of bacteria.

After the injection as stated above, the plunger 26 of the syringe B is immediately pulled to draw in the mixture of the coloring composition and the sample from the container E (such as a microtube) through the sample inlet 24 at the end of the sampling member C attached to the end of the syringe B, so that its filtration under suction may take place.

As a result of its filtration under suction, the dyed bacteria are collected on the hydrophobic filter 21. At the same time, any excess of coloring matter is removed. If the plunger 26 of the syringe B is pulled to produce suction, the solution containing the dyed sample (the mixed solution of the sample solution and the coloring composition D) is collected on the hydrophobic filter 21, while the excess of coloring matter is simultaneously removed.

The method of this invention as set forth in claim 10 enables a simple process of operation, since it is possible to carry out the collection of the dyed bacteria on the hydrophobic filter and the removal of any excess of coloring matter simultaneously as stated above.

According to the method of this invention as set forth in claim 10, the collection of the dyed bacteria on the hydrophobic filter 21 and the removal of the excess of coloring matter are carried out simultaneously as stated above, and the number of bacteria in the sample is determined from the degree of staining of the filter 21.

After the collection of the dyed bacteria on the filter 21, the sampling member C is detached from the end of the syringe B to expose the end of the filtering vessel A. The degree of staining of the bacteria on the hydrophobic filter 21 held at the end of the filtering vessel A, or the intensity of their color is compared with, for example, a color reference chart F prepared by employing samples containing known numbers of bacteria, whereby the number of bacteria in the sample is determined. The color reference chart F shown in Figures 5 and 6 is merely an example, and each of the numbers (③ to ⑦) marked on the chart means "log number per ml".

According to the method of this invention as set forth in claim 10, the collection of the dyed bacteria on the hydrophobic filter and the removal of the excess of coloring matter are carried out simultaneously as stated above, and the number of bacteria in the sample is determined from the degree of staining of the filter.

The number of bacteria in the sample is determined from the degree of staining of the bacteria in the sample from which the excess of coloring matter has been removed, as stated above. For determining the number of bacteria, (1) visual comparison with the color reference chart is the simplest way, but (2) it is alternatively possible to employ colorimetric analysis based on the measurement of optical density (O.D.), as already stated in the description of the invention as set forth in claim 1. For details of these methods for determining the number of bacteria, reference is made to the description of the invention as set forth in claim 1.

The invention will now be described in detail by way of examples embodying it.

Example 1:

(1) Preparation of a color reference chart for determining the number of lactic-acid bacteria:

A commercially available yogurt (product manufactured and sold by Zen-No under the tradename: Grated Apple) was diluted with water to make dilutions containing lactic-acid bacteria in the amounts of ① about 10^5/ml, ② about

10^6/ml, and ③ about 10^7/ml, respectively, and three standard samples were prepared from each dilution. The number of lactic-acid bacteria grown by culture was obtained by counting the number of lactic-acid bacteria grown by 72 hours of culture at 37°C on a BCP added plate count agar culture medium (product of Eiken Chemical).

The device shown in Figure 1 was used for experiments. The dimensions and reagents were as follows:

- Filtering vessel A: 5 mm O.D., 4 mm I.D. and 135 mm long; made of vinyl chloride;
- Coloring composition 5: 300 μl of a phosphoric acid buffer solution having a pH of 7, and containing 0.0002% of Fuchsine and 0.05% of Tween 20;
- Hydrophobic filter 6 for bacterial detection: A poly-tetrafluoroethylene membrane filter (having a pore diameter of 3 μm and a coloring area diameter of 1.5 mm);
- Filter support 7: A hollow plug formed from a silicone resin (4 mm O.D., 1.5 mm hole diameter, and 7 mm long).

Each standard sample in the amount of 100 μl was added to the coloring composition through the sample inlet, and the plunger 14 of the syringe 13 was pulled for filtration under suction. The collection of the dyed lactic-acid bacteria on the hydrophobic filter 6 and the removal of the excess of coloring matter were thereby carried out simultaneously. Visual examination was made of the degree of staining of lactic-acid bacteria on the hydrophobic filter 6 (or the degree of staining of circles having a diameter of 1.5 mm), and the results were as shown in Table 1 below.

Table 1

Standard sample	Degree of staining	Judgment (number/ml)
①	Weak (light pink)	About 10^5/ml
②	Medium (pink)	About 10^6/ml
③	Strong (red)	10^7/ml or more

Color photographs were taken of these stained filters as standard samples to prepare a color reference chart.

(2) Determination of the number of lactic-acid bacteria:

The device as shown in Figure 1 and used at (1) above was used for determining the number of lactic-acid bacteria in those samples of yogurt which had been stored in a refrigerator for two, three and four weeks, respectively. The dimensions of the device shown in Figure 1 and the reagents used were as stated at (1) above.

Three kinds of yogurt (products manufactured and sold by Zen-No under the tradenames: Grated Apple, Plain Type, and Grated Carrot) which had been stored in the refrigerator for certain periods of time were each diluted with water to 1000 times as large in volume, and each diluted yogurt in the amount of 100 μl was added to the device as shown in Figure 1 for filtration under suction. The collection of the dyed bacteria on the hydrophobic filter 6 and the removal of the excessive coloring matter were thereby carried out simultaneously. Visual examination was made of the degree of staining of lactic-acid bacteria on the hydrophobic filter 6 (or the degree of staining of circles having a diameter of 1.5 mm), and visual judgment was made by comparing the results with the color reference chart as prepared at (1) above.

The number of lactic-acid bacteria in each yogurt was determined by multiplying by 1000 (10^3) which was the number of times by which each yogurt had been diluted. Its determination took 60 seconds. The results are shown in Table 2.

For the sake of comparison, it also shows the number of lactic-acid bacteria as determined by culture (72 hours of culture at 37°C on a BCP added plate count agar culture medium made by Eiken Chemical).

Table 2

kind of yogurt	Method of determination	Number of lactic-acid bateria (number/ml)		
		After 2 weeks of storage	After 3 weeks of storage	After 4 weeks of storage
Grated apple	This invention	$\geqq 10^{10}$	10^9	10^8
	Culture	1.9×10^{10}	5.6×10^8	5.2×10^8
Plain type	This invention	$\geqq 10^{10}$	10^9	10^8
	Culture	4.1×10^{10}	8.1×10^8	9.3×10^7
Grated carrot	This invention	$\geqq 10^{10}$	10^9	10^8
	Culture	4.9×10^{10}	1.0×10^9	1.7×10^8

Example 2:

(1) Preparation of a color reference chart for determining the number of bacteria in urine:

Escherichia coli (ATCC 11303) was used for experiments. The urine which had been collected from a healthy male, and to which 0.1% of glucose had been added, was subjected to sterile filtration by a polytetrafluoroethylene (PTFE) membrane filter (made by Toyo Filter Paper) having a pore diameter of 0.5 μm to prepare a culture medium.

The medium was inoculated with the colon bacilli, and left to stand at 37 °C for 24 hours of culture to form a cultured bacterial solution as a bacterial suspension for standard samples. The same urine of the healthy male was filtered by a polytetrafluoroethylene membrane filter (made by Toyo Filter Paper) having a pore diameter of 0.5 μm to prepare a bacterial diluent.

Appropriate amounts of bacterial diluent were added to the bacterial suspension for a standard sample to prepare five standard samples having bacterial concentrations of ① about 1000/ml, ② about 10,000/ml, ③ about 100,000/ml, ④ about 1,000,000/ml, and ⑤ about 10,000,000/ml. The number of bacteria grown by culture was obtained by counting the number of colonies resulting from 24 hours of culture at 37°C on a CLED agar plate culture medium.

The device shown in Figure 2 was used for experiments. The dimensions and reagents were as follows:

- Filtering vessel A: 5 mm O.D., 4 mm I.D. and 135 mm long; made of vinyl chloride;
- Slender sampling tube 1: 2 mm O.D. and 1 mm I.D.; made of polyethylene;
- Prefilter 4: 4 mm O.D. and 4 mm I.D.; made of polyvinyl alcohol, and having a pore diameter of 60 μm;
- Coloring composition 5: 300 μl of a phosphoric acid buffer solution having a pH of 7, and containing 0.0002% of Fuchsine and 0.05% of Tween 20;
- Hydrophobic filter 6 for bacterial detection: A polytetrafluoroethylene membrane filter (having a pore diameter of 3 μm and a coloring area diameter of 1.5 mm);
- Filter support 7: A hollow plug formed from a silicone resin (4 mm O.D., 1.5 mm hole diameter, and 7 mm long);
- Piston 8: Made of konjak (devil's-tongue), and 5 mm long;
- Aqueous solution 9 drawn into the syringe: 800 μl of water.

The device was of such construction that the aqueous solution 9 was drawn into the syringe 13 under suction, and caused the piston 8 to move, whereby the dye composition 5 was filtered and the dyed bacteria were collected on the hydrophobic filter 6.

The standard sample (sample containing bacteria) was drawn into the sampling tube 1 up to the scale (100 μl), as shown in Figure 2, and after the sampling tube 1 had been lifted from the sample, the sample therein was drawn into the filtering vessel A.

Then, the plunger 14 of the syringe 13 was pushed to push back the dye composition 5 to its original position. As a result, the air which had been drawn in with the sample was forced out.

Then, the device was raised at the syringe 13 above its horizontal position to move bubbles, and was then lowered at the syringe 13 below its horizontal position to move the bubbles back to their original position. This movement of bubbles caused the dye composition 5 to mix with the sample and dye the bacteria.

Finally, the piston 8 was moved in the aqueous solution 9 in the filtering vessel A for filtration under suction with the syringe 13.

This operation allowed the collection of the dyed bacteria on the hydrophobic filter 6 and the removal of the excessive coloring matter to occur simultaneously. Visual examination was made of the degree of staining of the bacteria on the hydrophobic filter 6 (or the degree of staining of circles having a diameter of 1.5 mm), and the results were as shown in Table 3 below.

Table 3

Standard sample	Degree of staining	Judgment (number/ml)
①	Not stained	$\leqq 10^3$
②	Very weak (very light pink)	About 10^4
③	Weak (light pink)	About 10^5
④	Medium (pink)	About 10^6
⑤	Strong (red)	$10^7 \leqq$

Color photographs were taken of these stained filters as standard samples to prepare a color reference chart.

(2) Determination of the number of bacteria in urine:

Samples of urine were collected from healthy persons and patients of various diseases, and the device as shown in Figure 2 and used at (1) above was used for determining the number of bacteria in the urine of each person. The dimensions of the device as shown in Figure 2 and the reagents used were as stated at (1) above.

The urine of each of the healthy persons and patients was drawn into the sampling tube 1 up to the scale (100 μl), as shown in Figure 2, and after the sampling tube 1 had been lifted from the sample, the sample therein was drawn into the filtering vessel A.

Then, the plunger 14 of the syringe 13 was pushed to push back the dye composition 5 to its original position. As a result, the air which had been drawn in with the sample was forced out.

Then, the device was raised at the syringe 13 above its horizontal position to move bubbles, and was then lowered at the syringe 13 below its horizontal position to move the bubbles back to their original position. This movement of bubbles caused the dye composition 5 to mix with the sample and dye the bacteria.

Finally, the piston 8 was moved in the aqueous solution 9 in the filtering vessel A for filtration under suction with the syringe 13. This operation allowed the collection of the dyed bacteria on the hydrophobic filter 6 and the removal of the excessive coloring matter to occur simultaneously.

Visual examination was made of the degree of staining of the bacteria on the hydrophobic filter 6 (or the degree of staining of circles having a diameter of 1.5 mm), and visual judgment was made by comparing the results with the color reference chart as prepared at (1) above.

The determination took 60 seconds. The results are shown in Table 4. For the sake of comparison, it also shows the number of bacteria as grown on a CLED agar plate culture medium (by 24 hours of culture at 37°C).

Table 4

Urine sample	Judgment by the method of this invention (number/ml)	Number of bacteria as determined by culture on CLED agar plate (number/ml)
Healthy person A (male, age 56)	$\leqq 10^3$	5.0×10^2
Healthy person B (female, age 52)	$\leqq 10^3$	3.3×10^3
Patient A (of an infection of the urinary tract, age unknown)	about 10^6	2.3×10^6
Patient B (of diabetes, age unknown)	about 10^5	1.5×10^5
Patient C (of an infection of the urinary tract, age unknown)	about 10^6	1.0×10^7
Patient D (of cerebral infarction, age unknown)	about 10^4	4.8×10^4
Patient E (of urethral homorrhage, age unknown)	about 10^5	4.7×10^5

Comparative Example 1:

When the number of bacteria in urine was determined by the method described in Japanese Patent Application Laid-Open No. Hei 4-218392, it took three minutes.

Example 3:

Examination was made of the degree of staining on a hydrophobic filter for bacterial detection when coloring matter was employed in different concentrations for samples containing equal numbers of bacteria.

Experiments were conducted as described below by employing Escherichia coli (ATCC 11303) as bacteria. A culture medium composed of a bouillon of meat was inoculated with bacteria, and left to stand at 37 °C for 24 hours for culture, and a bacterial diluent obtained by filtering the urine of a healthy male through a polytetrafluoroethylene membrane filter (made by Toyo Filter Paper) having a pore diameter of 0.5 μm was added to the cultured solution to prepare coloring solutions having bacterial concentrations of 10^4/ml and 10^6/ml.

Visual examination was made of the degree of staining on a hydrophobic filter for bacterial detection by employing a kit of this invention and coloring compositions containing 0.00001 to 0.0005% of coloring matter (Fuchsine). The degree of staining as visually determined was compared with the color reference chart as prepared in Example 2 (Table 3). The results are shown in Table 5.

Table 5

concentration of coloring matter	Bacterial concentration of 10^4/ml		Bacterial concentration of 10^6/ml	
	Degree of staining	Judgment (number/ml)	Degree of staining	Judgment (number/ml)
0.0005%	Weak	10^5	Strong	$10^7 \leqq$
0.0004%	Very weak	10^4	Medium	10^6
0.0003%	Very weak	10^4	Medium	10^6
0.0002%	Very weak	10^4	Medium	10^6
0.0001%	Very weak	10^4	Medium	10^6
0.00005%	Very weak	10^4	Medium	10^6
0.00001%	Very weak	10^4	Weak	10^5

As is obvious from the results shown in Table 5, there was no coincidence between the degree of staining and the result of judgment when the coloring matter in the coloring composition had a concentration of 0.0005% or above, or below 0.00001%. It is also obvious from Table 5 that the preferred concentration of the coloring matter is in the range of 0.00015 to 0.0004%.

Example 4:

(1) Preparation of a color reference chart for determining the number of bacteria in urine:

Experiments were conducted as described below by employing Escherichia coli (ATCC 11303) and Staphylococcus aureus (IFO 3183) as bacteria.

A culture medium composed of a bouillon of meat was inoculated with bacteria, and left to stand at 37 °C for 24 hours to prepare a cultured solution as a bacterial suspension for standard samples. The urine of a healthy male was filtered by a polytetrafluoroethylene membrane filter (made by Toyo Filter Paper) having a pore diameter of 0.5 μm to prepare a bacterial diluent.

Appropriate amounts of bacterial diluent were added to the bacterial suspension for standard samples to prepare five standard samples having bacterial concentrations of ① about 1000/ml, ② about 10,000/ml, ③ about 100,000/ml, ④ about 1,000,000/ml, and ⑤ about 10,000,000/ml. The number of bacteria grown by culture was obtained by counting the number of colonies resulting from 24 hours of culture at 37°C on a CLED agar plate culture medium.

The kit shown in Figures 5 and 6 was used for experiments. The dimensions and coloring composition were as fol-

lows:

- Coloring composition D: A solution containing 0.0002% of Fuchsine, 0.85% of salt and 0.05% of Tween 20;
- Filtering vessel A: A polypropylene tube having an O.D. of 4.6 mm, an I.D. of 4 mm and a length of 20 mm;
- Sampling member C: A tip graduated for 10, 50 and 100 μl, and having an I.D. of 5 mm at its large end (product of Quality, U.S.A.);
- Microtube (container E for the coloring composition): A polypropylene container having a capacity of 1.5 ml (product of Quality, U.S.A.);
- Hydrophobic filter 21 for bacterial detection: A polytetrafluoroethylene membrane filter (having a pore diameter of 3 μm and a coloring area diameter of 1.5 mm);
- Filter support 22: A silicone tube having a length of 7 mm (and an O.D. of 4 mm and an I.D. of 1.5 mm);
- Dropping bottle 25: A product of polypropylene having a capacity of 10 ml;
- Prefilter 23: A product of polyvinyl alcohol having a diameter of 4 mm, a length of 4 mm and a pore diameter of 60 μm.

The syringe B having a capacity of 3 ml was attached to one end of the filtering vessel A, while the sampling member C holding the prefilter 23 therein was detachably fitted on the other end of the filtering vessel A (at which it had the hydrophobic filter 21 for bacterial detection), as shown in Figure 6, and 100 μl of sample containing bacteria as stated above was introduced through the sample inlet 24 at the distal end of the sampling member C by suction with the syringe B.

After the introduction of the sample by suction with the syringe B as stated above, it was forced out and injected into the container E (microtube) holding 350 μl (7 or 8 droplets) of coloring composition D therein.

After its injection, the plunger 26 of the syringe was immediately pulled to draw in for filtration the mixture of the coloring composition and the sample from the container E (microtube) through the sample inlet 24 at the distal end of the sampling member C attached to the end of the syringe B. This step of filtration under suction caused the collection of the dyed bacteria on the hydrophobic filter 21 and the removal of the excessive coloring matter to take place simultaneously.

After the collection of the dyed bacteria on the hydrophobic filter 21 as stated, the sampling member C was detached from the end of the syringe B to expose the end of the filtering vessel A, and visual examination was made of the degree of staining of the bacteria on the hydrophobic filter 21 at the end of the filtering vessel A, and the results were as shown in Table 6 below.

Table 6

Standard sample	Degree of staining and judgment			
	Escherichia coli	Judgment (number/ml)	Staphylococcus aureus	Judgment (number/ml)
①	None	$\leqq 10^3$	None	$\leqq 10^3$
②	Very weak	about 10^4	Very weak	about 10^4
③	Weak	about 10^5	Weak	about 10^5
④	Somewhat strong	about 10^6	Somewhat strong	about 10^6
⑤	Strong	about 10^7	Strong	about 10^7
No bacteria added	None	-	None	-

Color photographs were taken of these stained filters as standard samples to prepare a color reference chart. There was no substantial difference in the degree of staining between Escherichia coli and Staphylococcus aureus.

(2) Determination of the number of bacteria in urine:

The process as described at (1) above for the preparation of a color reference chart for determining the number of bacteria in urine was repeated on samples of urine of healthy persons and patients of various diseases, and visual judgment was made by comparing the results with the color reference chart as prepared at (1) above to determine the number of bacteria in each urine sample. The results are shown in Table 7.

For the sake of comparison, the table also shows the number of bacteria as determined by 24 hours of culture at

37°C on a CLED agar plate culture medium.

Table 7

Urine sample	Judgment by the method of this invention (number/ml)	Number of bacteria as determined by culture on CLED agar plate (number/ml)
Healthy person A (male, age 56)	$\leqq 10^3$	1.7×10^3
Healthy person B (female, age 52)	$\leqq 10^3$	3.0×10^3
Patient A (of an infection of the urinary tract, age and sex unknown)	10^7	6.8×10^7
Patient B (of an infection of the urinary tract, age and sex unknown)	10^5	4.2×10^5
Patient C (of liver cancer, age and sex unknown)	10^4	3.2×10^4
Patient D (of cystitis, female, age 53)	10^6	2.0×10^6

Example 5:

(1) Preparation of a color reference chart for determining the number of bacteria in a fermented milk beverage:

A commercially available yogurt (product manufactured and sold by Zen-No under the tradename: Grated Apple) was diluted with water to make dilutions containing bacteria in the amounts of ① about 10^5/ml, ② about 10^6/ml, and ③ about 10^7/ml, respectively, and three standard samples were prepared from each dilution. The number of bacteria as determined by culture was obtained by counting the number of colonies grown by 72 hours of culture at 37°C on a BCP added plate count agar culture medium (product of Eiken Chemical).

The kit as used in Example 4, but excluding the prefilter was employed for determining the number of bacteria, and the reagents, devices, and operation were as described in Example 4.

The process of Example 4 was repeated for dyeing 100 μl of each standard sample and filtering it under suction, and visual examination was made of the degree of staining of the bacteria on the hydrophobic filter 21, and gave the results as shown in Table 8 below.

Table 8

Standard sample	Degree of staining	Judgment (number/ml)
(1)	Weak (light pink)	About 10^5/ml
(2)	Somewhat strong (pink)	About 10^6/ml
(3)	Strong (red)	About 10^7/ml

Color photographs were taken of these stained filters as standard samples to prepare a color reference chart.

(2) Determination of the number of bacteria in fermented milk beverages:

Three kinds of yogurt (products manufactured and sold by Zen-No under the tradenames: Grated Apple, Plain Type, and Grated Carrot) which had been stored in a refrigerator for certain periods of time were each diluted with water to 1000 times as large in volume to prepare samples.

The process as described above at (1) for the standard samples was repeated for dyeing 100 μl of each sample and filtering it under suction. As a result, the collection of the dyed bacteria on the hydrophobic filter 21 and the removal of the excessive coloring matter were carried out simultaneously. Visual examination was made of the degree of staining of the bacteria on the filter 21, and visual judgment was made by comparing the results with the color reference chart as prepared at (1) above.

The number of bacteria in each yogurt was determined by multiplying the results by 1000 (10^3) which was the

number of times by which each yogurt had been diluted. Its determination took 60 seconds. The results are shown in Table 9.

For the sake of comparison, the table also shows the number of bacteria as determined by culture (72 hours of culture at 37 °C on a BCP added plate count agar culture medium made by Eiken Chemical).

Table 9

Kind of yogurt	Method of determination	Number of bacteria (number/ml)			
		Initial	After 2 weeks of storage	After 3 weeks of storage	After 4 weeks of storage
Grated Apple	This invention	$\geqq 10^{10}$	$\geqq 10^{10}$	10^9	10^8
	Culture	3.0×10^{10}	1.9×10^{10}	5.6×10^8	5.2×10^8
Plain Type	This invention	$\geqq 10^{10}$	$\geqq 10^{10}$	10^9	10^8
	Culture	6.5×10^{10}	4.1×10^{10}	8.1×10^8	9.3×10^7
Grated Carrot	This invention	$\geqq 10^{10}$	$\geqq 10^{10}$	10^9	10^8
	Culture	8.0×10^{10}	4.9×10^{10}	1.0×10^9	1.7×10^8

The methods of this invention as set forth in claims 1 and 8 make it possible to carry out the collection of dyed bacteria on a hydrophobic filter and the removal of any excessive coloring matter simultaneously in a single step of filtering operation and thereby determine the number of bacteria in a sample quickly and easily. The methods of this invention as set forth in claims 1 and 8 make it possible to obtain results usually within one minute (in about 30 seconds after experience has been gained), and the method as set forth in claim 1 enables a particularly quick determination.

The methods of this invention as set forth in claims 1 and 8 make it possible to determine the number of bacteria in a sample without requiring any expert skill, or knowledge.

Moreover, the methods of this invention as set forth in claims 1 and 8 make it possible to check the presence of bacteria without relying upon culture, and can, therefore, be utilized for the screening of samples for a bacteriological examination in a hospital to thereby reduce the relevant costs of the patients and hospital.

The methods of this invention as set forth in claims 1 and 8 can be carried out even in a small hospital, or clinic, or even by any ordinary person, since they do not require any special apparatus.

Moreover, the methods of this invention as set forth in claims 1 and 8 have a very wide scope of use, since they are applicable to many kinds of bacteria if a hydrophobic filter having an appropriate pore diameter is employed.

The devices of this invention for determining the number of bacteria as set forth in claims 2 and 9 enable a very simple process which does not involve any such work as taking a sample by a dropper, or dropping a reagent from a reagent bottle, but for which it is sufficient to use a syringe for filtration. They do not require any skill, or any special equipment, but enable determination to be carried out at any place.

The device of this invention as set forth in claim 9 is a disposable one of simple construction, but enables the reuse of a syringe, since it ensures that no sample enter the syringe and cause its bacterial, or like contamination. The device of this invention as set forth in claim 8 is intended mainly for determining the number of lactic-acid bacteria, and enables the reuse of a syringe, since it is unlikely to bring about any serious problem of bacterial contamination.

The devices of this invention as set forth in claims 2 and 9 can advantageously be used at any site, since they are very simple, and inexpensive, and do not require any special apparatus. It is particularly beneficial that any ordinary person, or layman can use the device easily to inspect the quality of any water in his environment, such as river, pond, sea or hot-spring water, and thereby contribute to the preservation of a sound environment, and also to even examine the number of bacteria in liquid or solid, or other food.

Moreover, the devices of this invention as set forth in claims 2 and 9 can be used as a container for transporting to an examination room any sample containing bacteria to be identified after their quantitative analysis, if the slender tube is closed by heating, or otherwise, as the sample can be held in the device under suction.

The inventions as set forth in claims 2 and 9 can be said to be somewhat more complicated than those as set forth in claims 1 and 9, which are very simple, since they are intended for enabling the reuse of a syringe without giving rise to any recently controversial problem, such as bacterial contamination.

The method of this invention as set forth in claim 10 can determine the number of bacteria in a sample quickly and easily, since it does not require any dropper, or like device for taking a sample, but can carry out the collection of dyed bacteria on a hydrophobic filter and the removal of any excessive coloring matter simultaneously in a single step of fil-

tering operation under suction with a syringe. The method of this invention as set forth in claim 10 makes it possible to obtain results usually within one minute (and in about 30 seconds after experience has been gained).

The method of this invention as set forth in claim 10 makes it possible to determine the number of bacteria in a sample without requiring any expert skill, or knowledge.

Moreover, the method of this invention as set forth in claim 10 makes it possible to check the presence of bacteria without relying upon culture, and can, therefore, be utilized for the screening of samples for a bacteriological examination in a hospital to thereby reduce the relevant costs of the patients and hospital.

The method of this invention as set forth in claim 10 can be carried out even in a small hospital, or clinic, or even by any ordinary person, since they do not require any special apparatus.

The method of this invention as set forth in claim 10 has a very wide scope of use, since it is applicable to many kinds of bacteria if a hydrophobic filter having an adequate pore diameter is employed.

The method of this invention as set forth in claim 10 contributes to the proper control of food quality, since it makes it possible to ascertain the number of bacteria in food (such as yogurt) before its shipment.

The kits of this invention for determining the number of bacteria as set forth in claims 17 and 18 enable a very simple process which does not involve any such work as taking a sample by a dropper, or dropping a reagent from a reagent bottle, but for which it is sufficient to use a syringe for filtration. They do not require any skill, or any special equipment, but enable determination to be carried out at any place.

The kits of this invention as set forth in claims 17 and 18 can advantageously be used at any site, since they are very simple, and inexpensive, and do not require any special apparatus. It is particularly beneficial that any ordinary person, or layman can use the kits easily to inspect the quality of any water in his environment, such as river, pond, sea or hot-spring water, and thereby contribute to the preservation of a sound environment, and also to even examine the number of bacteria in liquid or solid, or other food.

Moreover, the kits of this invention as set forth in claims 17 and 18 can be used as a container for transporting to an examination room any sample containing bacteria to be identified after their quantitative analysis, if the slender tube is closed by heating, or otherwise, as the sample can be held in the device under suction.

Posibility of Industrial Utilization:

This invention can, thus, be used for examination in a wide variety of fields including urine analysis (for diagnosis) and metal processing.

Claims

1. A method of determining the number of bacteria in a sample quickly which comprises introducing a sample containing bacteria into a tubular filtering vessel holding therein a hydrophobic filter for bacterial detection, a coloring composition on that side of said filter where the sample is introduced into said vessel, and a support for said filter on the opposite side of said filter from said composition, dyeing the bacteria, filtering the sample by suction from said support to collect the dyed bacteria on said filter and remove the excess of coloring matter, and determining the number of the bacteria in the sample from the degree of staining of said filter.

2. A method of determining the number of bacteria in a sample quickly which comprises introducing a sample containing bacteria into a tubular filtering vessel holding therein a hydrophobic filter for bacterial detection, a coloring composition on that side of said filter where the sample is introduced, and a support for said filter, a piston and an aqueous solution on the opposite side of said filter from said composition, dyeing the bacteria, filtering the sample by moving said piston in said aqueous solution to collect the dyed bacteria on said filter and remove the excess of coloring matter, and determining the number of the bacteria in the sample from the degree of staining of said filter.

3. A method as set forth in claim 1, wherein said sample is of fermented milk, or a lactic-acid beverage.

4. A method as set forth in claim 2, wherein said sample is of urine.

5. A method as set forth in claim 1 or 2, wherein said composition comprises coloring matter, a buffer solution and a surface active agent.

6. A method as set forth in claim 1 or 2, wherein said composition contains coloring matter at a concentration of 0.00001 to 0.00045% (w/v).

7. A method as set forth in claim 1, 2 or 6, wherein said composition contains a surface active agent at a concentration

of 0.001 to 1.0% (w/v).

8. A device for determining the number of bacteria which comprises a tubular filtering vessel having a sample inlet at one end, while the other end thereof enables suction with a syringe, said vessel holding a coloring composition, a hydrophobic filter for bacterial detection and a filter support therein in their order as viewed from said sample inlet.

9. A device for determining the number of bacteria which comprises a tubular filtering vessel having a slender sampling tube connected at one end, while the other end thereof enables suction with a syringe, said vessel holding a prefilter, a coloring composition, a hydrophobic filter for bacterial detection, a filter support, a piston movable in said vessel, an aqueous solution and a plug preventing the leakage of said aqueous solution therein in their order as viewed from said slender tube.

10. A method of determining the number of bacteria in a sample quickly which comprises introducing a sample containing bacteria by suction into a filtering vessel holding a hydrophobic filter for bacterial detection therein, extruding it into a coloring composition, filtering it by suction to collect the dyed bacteria on said filter and determining the number of the bacteria in the sample from the degree of staining of said filter.

11. A method as set forth in claim 10, wherein said composition comprises coloring matter, a buffer solution and a surface active agent.

12. A method as set forth in claim 10, wherein said composition contains coloring matter at a concentration of 0.00001 to 0.00045% (w/v).

13. A method as set forth in claim 10 or 12, wherein said composition contains a surface active agent at a concentration of 0.001 to 1.0% (w/v).

14. A method as set forth in claim 10, wherein said vessel has one end to which a sampling member holding a prefilter therein is attached.

15. A method as set forth in any of claims 10 to 14, wherein said sample is of urine.

16. A method as set forth in claim 10, wherein said sample is of fermented milk, or a fermented milk beverage.

17. A kit for determining the number of bacteria which comprises a filtering vessel holding a hydrophobic filter for bacterial detection and a filter support therein, a sampling member containing a prefilter and adapted for connection to one end of said vessel, a coloring composition, a container for said composition, and a color reference chart.

18. A kit for determining the number of bacteria which comprises a filtering vessel holding a hydrophobic filter for bacterial detection and a filter support therein, a sampling member adapted for connection to one end of said vessel, a coloring composition, a container for said composition, and a color reference chart.

FIG.1

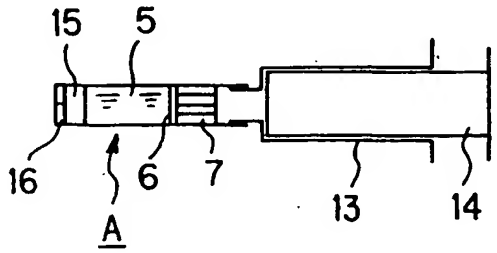


FIG.2

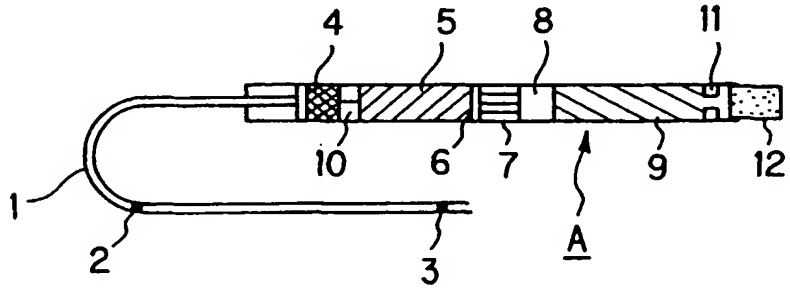


FIG.3

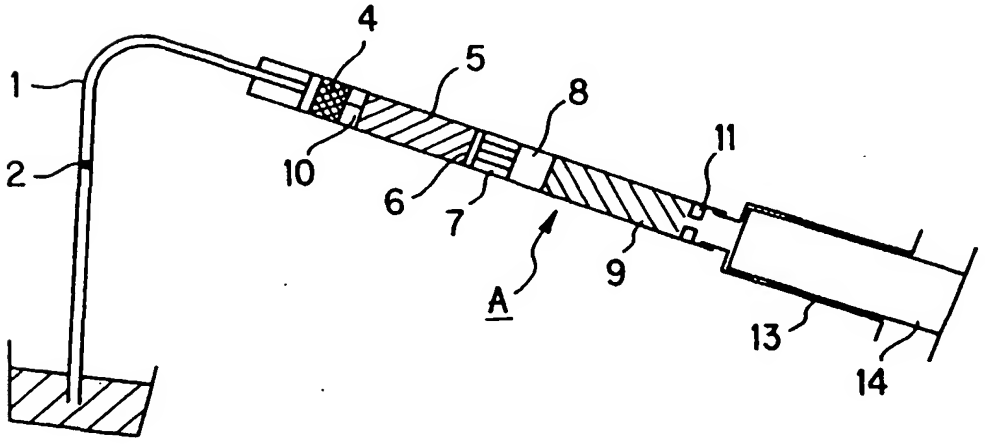


FIG.4a

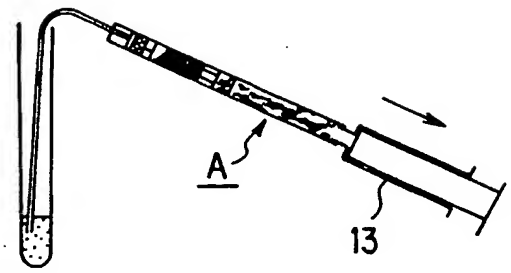


FIG.4b

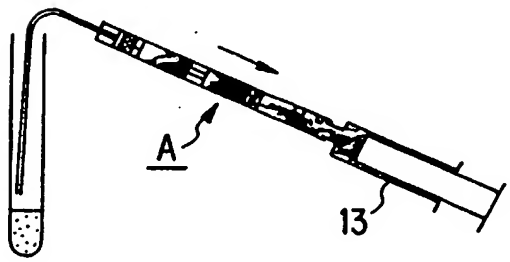


FIG.4c

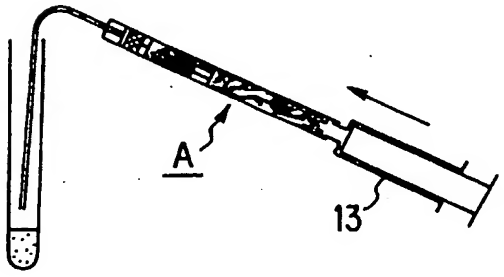


FIG.4d

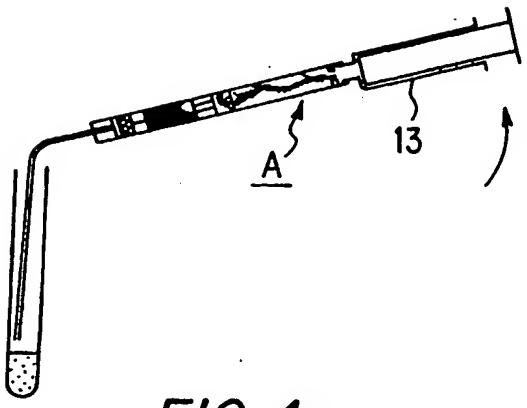


FIG.4e

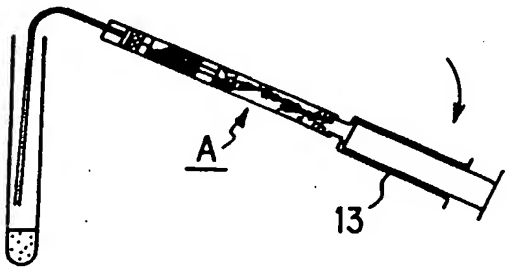


FIG.4f

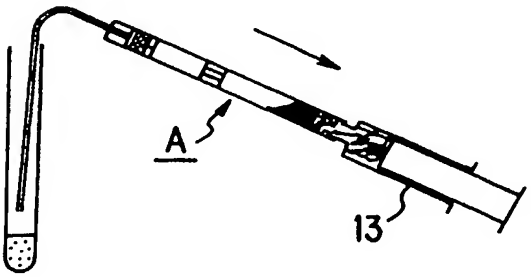


FIG.5

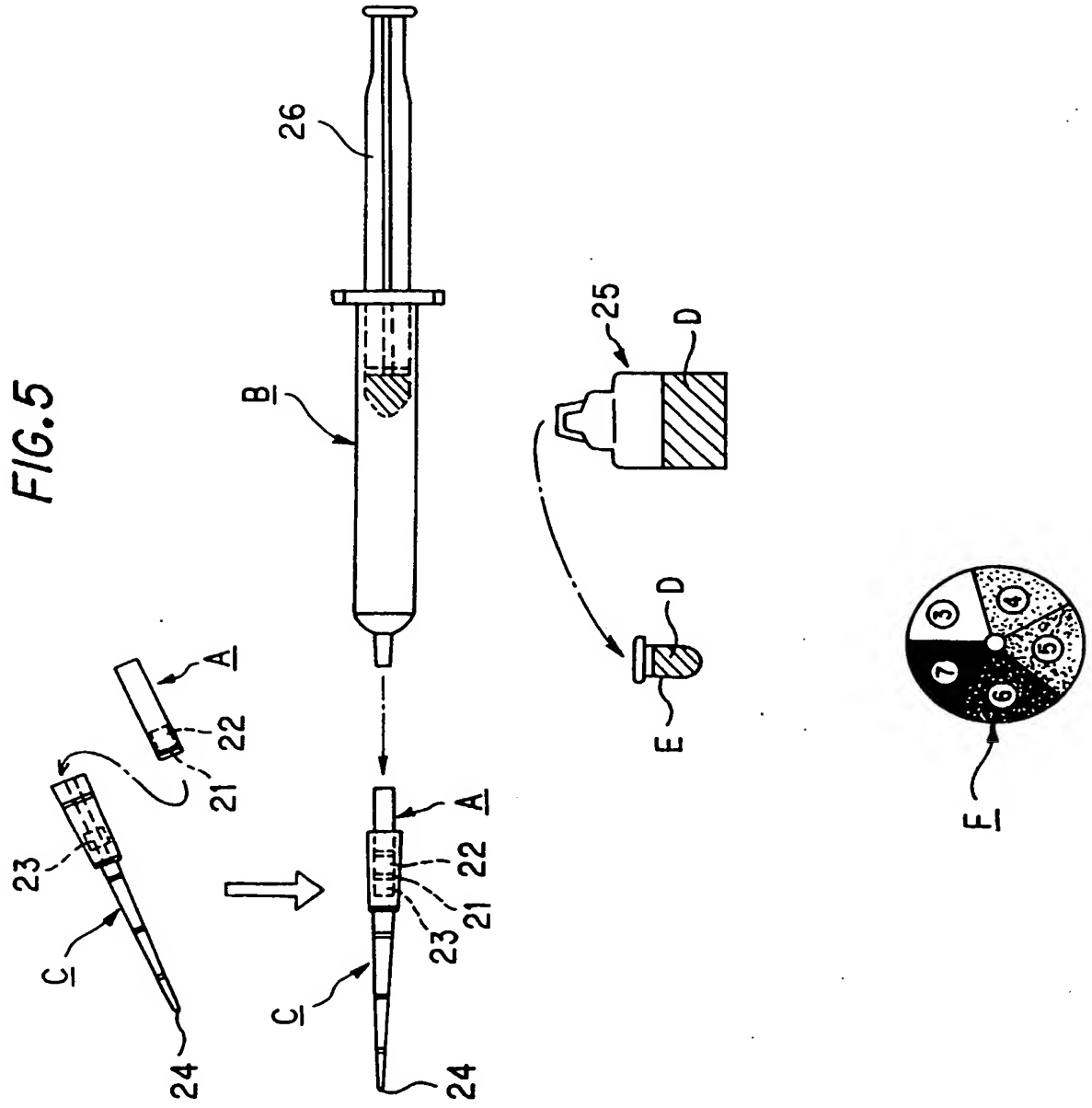
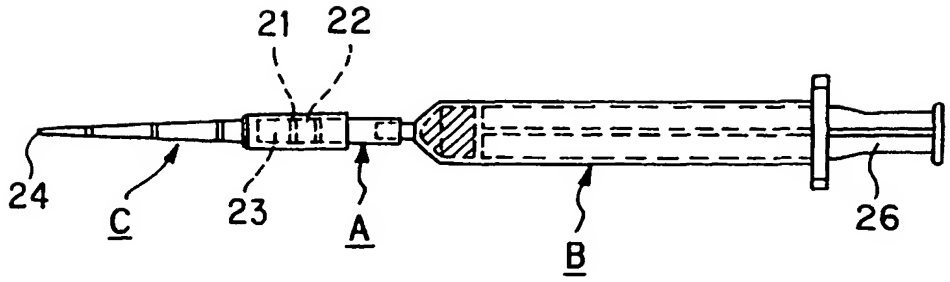
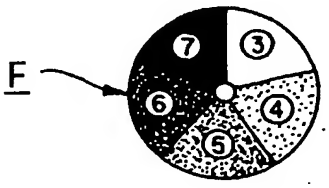


FIG.6





INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/00815

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^6 C12Q1/06, C12M1/34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
Int. Cl^6 C12Q1/06, C12M1/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 4-218392, A (Idemitsu Kosan Co., Ltd.), August 7, 1992 (07. 08. 92) & EP, 465987, A & US, 5403720, A	1 - 18
A	Edited by Kyoto University Rokuseikai "New edit. Agricultural Chemistry experiment Book (vol. 2)", April 10, 1957 (10. 04. 57), Sangyo Tosho (Tokyo), p.428-429	1 - 9, 17 - 18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"E" earlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
April 22, 1996 (22. 04. 96)	May 14, 1996 (14. 05. 96)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)